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Discovery of Baeyer–Villiger monooxygenases from photosynthetic eukaryotes

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ABSTRACT

Baeyer–Villiger monooxygenases are attractive "green" catalysts able to produce chiral esters or lactones starting from ketones. They can act as natural equivalents of peroxyacids that are the catalysts classically used in the organic synthesis reactions, consisting in the cleavage of C—C bonds with the concomitant insertion of an oxygen atom.

In this study, two type I BVMOs have been identified for the first time in photosynthetic eukaryotic organisms, the red alga *Cyanidioschyzon merolae* (Cm) and the moss *Physcomitrella patens* (Pp). A biocatalytic characterization of these newly discovered enzymes, expressed in recombinant forms, was carried out. Both enzymes could be purified as holo enzymes containing a FAD cofactor. Their thermostability was investigated and revealed that the Cm-BVMO is the most thermostable type I BVMO with an apparent melting temperature of 56 °C. Substrate profiling revealed that both eukaryotic BVMOs accept a wide range of ketones which include aromatic, aliphatic, aryl aliphatic and bicyclic ketones. In particular, linear aliphatic ketones (C9 and C12), carrying the keto functionality in different positions, resulted to be the best substrates in steady state kinetic analyses. In order to restore the BVMO-typifying sequence motif in the Pp-BVMO, a mutant was prepared (Y160H). Intriguingly, this mutation resulted in higher activities on most tested substrates. The recombinant enzymes displayed k_{cat} values in the 0.1–0.2 s^{-1} range, which is relatively low when compared with other known type I BVMOs. This may hint to a role in secondary metabolism in these photosynthetic organisms, though their exact function remains to be established.

1. Introduction

The Baeyer–Villiger reaction is an important oxidative reaction in organic synthesis firstly reported by Adolf von Baeyer and Victor Villiger in 1899 [1]. In this reaction, ketones are oxidized into the corresponding esters or lactones by peroxyacids resulting in oxygen insertion next to the carbonyl group. Reagents able to carry out the Baeyer–Villiger reaction are peroxyacids such as meta-chloroperoxybenzoic acid, trifluoroperoxyacetic acid, peroxyacetic acid, hydrogen peroxide and others. Such molecules are expensive, toxic and hazardous; they are strong oxidants and react with other functional groups [2]. The reaction mechanism consists in the nucleofilic attack of peroxyacid to the carbonyl function of the substrate forming a tetrahedral intermediate called the "Criegee intermediate", which undergoes rearrangement to the corresponding ester or lactone; one of the group attached to the carbonyl carbon migrates onto the electron deficient oxygen atom with the simultaneous dissociation of the O—O bond. The regiochemistry of the reaction depends on the relative migratory ability of the substituents attached to the carbonyl group and on the stereoelectronic features of the substrate [3]. Moreover, if the migrating carbon is chiral the stereochemistry is retained [4].

Baeyer–Villiger monooxygenases (BVMOs) are flavin-containing monooxygenases able to catalyze a remarkable wide variety of oxidative reactions [5], which are difficult to obtain chemically: the nucleophilic oxidation of carbonyl groups but also the electrophilic oxidation of heteroatoms such as sulphur [6,7], nitrogen [8] and boron [9], with good enantioselectivity. Complete enantioselectivity has been reported for the epoxidation of few selected olefin substrates too [10]. Indeed, the great potential of these enzymes is the ability to produce chiral lactones and sulfoxides by asymmetric synthesis and this makes them attractive for application in industrial organic synthesis [11,12].

BVMOs contain a flavin cofactor, FAD or FMN, not covalently but tightly bound to the enzyme. This flavin cofactor has to be activated by electron donors, NADH or NADPH, which give reduction

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equivalents allowing molecular oxygen binding. Depending on cofactor usage, BVMOs can be classified into two types [13]. Type I BVMOs are FAD and NADPH dependent and contain two-dinucleotide binding domains (βαβ-folds) known as Rossman motifs, one for the FAD binding and the other one for the NADPH binding. They are composed of only one polypeptide. Type II BVMOs are FMN and NADH dependent and consist of two distinct subunits, a dehydrogenase using NADH to reduce FMN and a second subunit able to perform the Baeyer–Villiger reaction using the reduced flavin.

There is another monooxygenase, MtmOIV, able to perform the Baeyer–Villiger reaction, involved in mithramycin biosynthesis. Mithramycin is a polyketide anticancer antibiotic produced by the soil bacterium *Streptomyces argillaceus* (ATCC 12956) and other streptomycetes [14]. Sequence analysis and crystal structure revealed that MtmVIO can not be classified as a type I or type II BVMOs but it appears to be an atypical BVMO belonging to a different flavoprotein monooxygenase family [15].

The best-characterized BVMOs are those of type I, which display good substrate promiscuity and are therefore attractive for synthetic applications [16]. In particular, the most famous enzymes are cyclohexanone monooxygenase (CHMO) from *Acinetobacter* NCIMB 9871 and phenylacetone monooxygenase (PAMO) from *Thermobifida fusca*. The former enzyme was also the first one to be described, in the middle seventies [17]. Up to ten years ago, however, few BVMOs had been cloned and overexpressed. Thanks to the vast genetic information that has become available by genome sequencing, the number of recombinant BVMOs has increased considerably. In particular, the technique of genome mining has proved to be an efficient approach to discover new biocatalysts. Previous studies performed by this approach showed that type I BVMOs were present in a vast variety of bacteria and fungi, but no representative was found in Archea, plants or human genomes [18].

In order to find novel and promising biocatalysts, we used the genome mining approach to uncover BVMOs within specific and unusual organisms. Using the sequence of a prototype BVMO, phenylacetone monooxygenase (PAMO), as template and the "fingerprint" motif for type I BVMOs (FxGxxxHxxxWP/D; [19]) as discriminant, we have identified two new putative BVMO-encoding genes in the red alga *Cyanidioschyzon merolae* (Cm) and in the moss *Physcomitrella patens* (Pp). Photosynthetic eukaryotes appear as very uncommon sources for BVMOs. In fact, only two BVMOs from eukaryotic origin have been cloned and expressed to date. Both BVMOs were derived from fungi: the ascomycetes *Cylindrocarpon radicicola* ATCC 11011 [20] and *Aspergillus fumigatus* Af293 [21]. The discovery of BVMOs in photosynthetic eukaryotes is novel and may provide new biocatalytic features.

2. Experimental

2.1. Organisms and culture conditions

Cyanidioschyzon merolae strain 10D (NIES-1332) was obtained from the Microbial Culture Collection of the National Institute for Environmental Studies, Tsukuba, Japan. Cultures of *C. merolae* were grown at 30 °C in M-Allen medium (http://mcc.nies.go.jp), in 500 ml flasks on a rotatory platform shaker at 70 rpm. Light conditions used were 25–27 μmol photons m^{-2} sec^{-1}.

E. coli strains (XL1-blue and BL21 (DE3)) were routinely cultured in LB media and supplemented with the antibiotic kanamycin (50 μg/ml).

The starting culture of *Physcomitrella patens* was kindly provided by Dr. Tomas Morosinotto (Department of Biology, University of Padova). It was cultivated by micro propagation in $PpNH_4$ rich medium: 0.8 g/l $CaNO_3$ $4H_2O$, 0.25 g/l $MgSO_4$ $7H_2O$, 0.0125 g/l $FeSO_4$ $7H_2O$, 500 mg/l NH_4 tartrate, 5 g/l glucose, 1 ml KH_2PO_4/KOH buffer pH 7, and 1 ml trace solution elements per liter. KH_2PO_4/KOH buffer contained 25 g KH_2PO_4 per 100 ml of milliQ water; pH 7 was obtained by titrating with 4 M KOH. Trace element solution contained 55 mg/l $CuSO_4$ $5H_2O$, 55 mg/l $ZnSO_4 \cdot 7H_2O$, 614 mg/l H_3BO_3, 389 mg/l $MnCl_2 \cdot 4H_2O$, 55 mg/l $CoCl_2 \cdot 6H_2O$, 28 mg/l KI, 25 mg/l $Na_2MoO_4 \cdot 2H_2O$. Cultures were grown in a growth chambers at 24 °C, with 16 h light/8 h dark photoperiod and a light intensity of 40–50 μE. Nutrition media were autoclaved for 20 min at 121 °C and plant agar (7 g/l) was added in order to prepare solid media.

2.2. Reagents and enzymes

All chemicals were purchased from Fluka and Sigma–Aldrich (Milan, Italy). Restriction enzymes were obtained from New England Biolabs (NEB) or from Promega; Phusion™ High-fidelity DNA polymerase from Finnzymes; TSAP (Thermosensitive Alkaline Phosphatase) from Promega; T4 DNA Ligase from NEB. IMAC-Select Affinity Gel resin was purchased from Sigma–Aldrich.

2.3. Sequence analysis and cloning

The NCBI resource was used for DNA sequence analysis; searches and multiple alignments of BVMO sequences were respectively produced by programs BLAST and ClustalW.

Expression vectors were produced by digestion of pET-28a(+) with *NcoI*/*NotI* and ligation of the amplified Cm-BVMO and Pp-BVMO sequences, cut by the same enzymes. These sequences were obtained by two subsequent PCR reactions, the first one producing a preliminary "large" amplimer, which was then used for the second amplification, by nested mutagenic primers. The following synthetic oligonucleotides were used for PCR amplification of the target gene from *C. merolae* genomic DNA: external primer pair, forward 5′-AGTGATGCGCGTGGCCGGCA-3′ and reverse 5′-AGGTGTCTGCACCTCGCCAGCG-3′; nested primer pair, forward 5′-TTTGACCGGCCATGGGAGCGGAGCTCAAC-3′ and reverse 5′-GCATCCACCGCGCCGGCGTACAGCGAAG-3′. The following synthetic oligonucleotides were used for PCR amplification of the target gene from *P. patens* genomic DNA: external primer pair, forward 5′-ACAGGCCACGGGGGTAGTTCTGTTG-3′ and reverse 5′-CAACCCTGGACAGCATCGGAAGCCT-3′; nested primer pair, forward 5′-AAGTATGTCCAATTCCATGGC-TGAGTTCGATGCTGTTATAGTCGGAG-3′ and reverse 5′-AAACAAT-GCCCGCGGCCGCCAGCTTGAATCCC-3′. The sequence variant Y160H was constructed using the QuikChange® II Site-Directed Mutagenesis Kit of Stratagene using plasmid pET28_Pp-BVMO as template with the oligonucleotide 5′-GGCTCATCGTACCA-CACGGGC-3′ and its complementary one.

2.4. Expression, analysis and purification of recombinant proteins

The recombinant enzymes, hereafter called Cm-BVMO, Pp-BVMO and Pp-Y160H-BVMO, were expressed in *E. coli* BL21 (DE3) or *E. coli* ARCTIC Express® (Stratagene). Pre-cultures were carried out in 5 ml LB medium at 37 °C containing 50 μg/ml kanamycin. Larger cultures were carried out in 1 L LB medium with riboflavin addition (100 μM). The cells were grown in a shaking incubator at 37 °C to an optical density at 600 nm (OD_{600}) of 0.4–0.6, then induced by addition of IPTG to a final concentration of 0.2 mM and cultivated at 18 °C overnight. The cells were harvested by centrifugation (4 °C, 10 min, 4500 × *g*) and washed with Tris/HCl buffer (Tris 50 mM, pH 8.0). Cell disruption was obtained by French Press and crude extract was centrifuged (4 °C, 20 min, 15,000 × *g*) to separate soluble e insoluble fractions. FAD cofactor (at 100 μM final concentration) was added to the crude extract before cell disruption.

Expression of recombinant protein was checked by SDS-PAGE analysis. The identity of the proteins was verified by immunoblotting using anti His-tag antibodies. Overexpressed proteins were purified by immobilized-metal affinity chromatography (IMAC). Soluble fractions obtained from 1 l culture were incubated with the resin for 1 h at 4 °C and then loaded on a 5 ml column (Bio-Rad). The column was washed by gravitational flow with three column volumes of 50 mM Tris/HCl, 300 mM NaCl buffer, then with two column volumes of the same buffer with added 5 mM imidazole. Elution was performed by five column volumes of 50 mM Tris/HCl, 300 mM imidazole solution. Enzyme concentration was measured by spectrophotometry, measuring concentration of free flavin in a solution of denatured protein and calculated as previously reported [22].

2.5. Activity assay, kinetics and ThermoFAD measurements

BVMO activity is usually determined spectrophotometrically by monitoring the consumption of NADPH at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$). Reaction mixtures (1 ml) contained 50 mM Tris–HCl buffer pH 8.0, 100 μM NADPH, 0.4 μM pure enzyme, and 10 μl of 1 mM of *rac*-bicyclo[3.2.0]hept-2-en-6-one in dioxane. Adding the enzyme to the mixture started the reaction. One unit of BVMO is defined as the amount of protein that oxidizes 1 μmol NADPH per minute.

Steady-state kinetic parameters of the different substrates were determined using purified enzymes and substrate concentrations ranging from 0 to 5 mM. Data were fitted using the Michaelis–Menten equation by the program SigmaPlot.

The apparent unfolding temperatures of the recombinant enzymes, *Tm*s, were determined using the *Thermo*FAD method [23]. 30 μl of 1 mg/mL protein in Tris-HCl buffer (50 mM, pH 7.5) were loaded in a Real Time PCR machine (Eppendorf) fitted with a 470–543 nm excitation filter and a SYBR Green emission filter (523–543 nm). A temperature gradient from 20 to 90 °C was applied (1 °C/min), and fluorescence data were recorded. A sigmoidal curve was obtained after plotting the fluorescence amount against the temperature. The *Tm* values were determined as the maximum of the derivative of the obtained sigmoidal curve

2.6. Substrate screening

The phosphate activity assay was performed according to the protocol reported by Riebel [24]. All substrates were dissolved in dioxane (maximum 5%) and tested in 96 wells plates.

2.7. Conversions and GC/GC–MS analysis

For GC and GC–MS analysis, samples of 500 μl 50 mM Tris–HCl (pH 7.5) containing 2 mM substrate, 5% dioxane, 100 μM NADPH, 3.0 μM PTDH, 10 mM phosphite and 1 μM BVMO were incubated shaking at 25 °C from 1 to 20 h. The reactions were stopped by extraction with ethyl acetate (3 × 0.5 ml, including 0.1% mesitylene as an internal standard), dried with magnesium sulfate and analyzed directly by GC or GC–MS [5] to determine the degree of conversion.

3. Results

3.1. Two new BVMOs from photosynthetic eukaryote organisms

By using the protein sequence of PAMO as template for a tBLASTn search and the consensus motif FxGxxx**H**xxxWP [19] as discriminant (allowing just single conservative mutation), two new putative type I BVMO enzymes were identified from two photosynthetic eukaryotes: *Cyanidioschyzon merolae* (a red alga) and *Physcomitrella patens* (a moss). Both identified BVMO sequences also contained other conserved regions, such as two Rossmann fold motifs (GXGXX(G/A)) that are known to be involved in dinucleotide cofactor binding [25] and the recently annotated motif [A/G]GxWxxxx[F/Y]P[G/M]xxxD, which includes residues of the active site and therefore represents a BVMO-typifying consensus sequence [24]. A ClustalW alignment of the two proteins with PAMO and cyclohexanone monooxygenase (CHMO) from *Acinetobacter* NCIMB 9871 is shown in Fig. 1.

With the aim of considering the evolutionary relationships of the two proteins with other known BVMOs, we selected a set of 24 prototype enzymes among those that are well characterized as recombinant products and representative for substrate specificities. We aligned thus 26 protein sequences and produced the branching diagram shown in Fig. 2. It resulted that the sequence from *C. merolae* most likely shares a common ancestor with acetone monooxygenase (ACMO) from the actinobacterium *Gordonia* sp. TY-5 and methyl ketone monooxygenase (MEKMO) from the proteobacterium *Pseudomonas veronii*, strain MEK700. The protein of *P. patens* forms a cluster with two other enzymes of actinobacterial origin: PAMO and steroid monooxygenase (STMO) from *Rhodococcus rhodochrous*. The observed homologies were considered for the discussion below.

The intron-less coding gene for the putative BVMO from *C. merolae* is present in chromosome 12 of the organism. The primary structure of the translated protein, which is annotated in GenBank as a steroid monooxygenase (BAM80902.1), shares 43% identity with the sequence of PAMO, 37% with cyclohexanone monooxygenase (CHMO) from *Acinetobacter* NCIMB 9871 and 50% with CHMO from *Rhodococcus* Phi1.

The 536 amino acids long putative enzyme from *P. patens* (predicted protein XP_001758613.1 in the NCBI data bank) is also coded by a continuous open reading frame of the nuclear genome; it shows 54% sequence identity with PAMO and 44% with CHMO from *Acinetobacter* NCIMB 9871. One of the two fingerprint motifs for type I BVMO is, however, not strictly conserved but differs in one amino acid: FxGxxx**Y**xxxWP instead of FxGxxx**H**xxxWP (Fig. 1). This is noteworthy, since it is known that the central histidine of this consensus sequence (His173 in PAMO) is involved in catalysis and FAD binding [26]. For this reason we planned to study the wild-type form of the putative flavoenzyme in comparison with a mutant form in which the consensus motif would be artificially repaired.

The identified coding sequences together with the variant obtained by site-directed mutagenesis were cloned into the pET28a(+) expression vector, permitting their translation in the form of fusion proteins with carboxy-terminal hexahistidine tags. The recombinant enzymes were designated Cm-BVMO (from *C. merolae*), and Pp-BVMO (from *P. patens*) and Pp-Y160H-BVMO.

Overexpression tests were then performed in *E. coli* BL21(DE3) strain and parameters such as temperature and IPTG concentration were optimized. In all cases the proteins were expressed at very high level and were mostly present in the cell debris after cell homogenization, indicating insolubility. To overcome this problem, different known strategies were unsuccessfully tried: expression at low temperature in a bacterial strain containing cold-adapted chaperones (*E.coli* ARCTIC Express®) or fusion with partners favoring solubility (bacterial MBP and yeast SUMO) (data not shown). Only upon addition to the culture medium of an excess of riboflavin, the precursor of FAD, bright yellow colored recombinant enzymes could be recovered in the soluble fraction of the homogenate. This result underlines the requirement of the flavin cofactor for correct folding of BVMOs.

The proteins were purified to homogeneity by IMAC chromatography (Fig. 3); the evaluated yields were 45 mg for Cm-BVMO, 9.5 mg for Pp-BVMO and 10 mg for Pp-Y160H-BVMO per liter

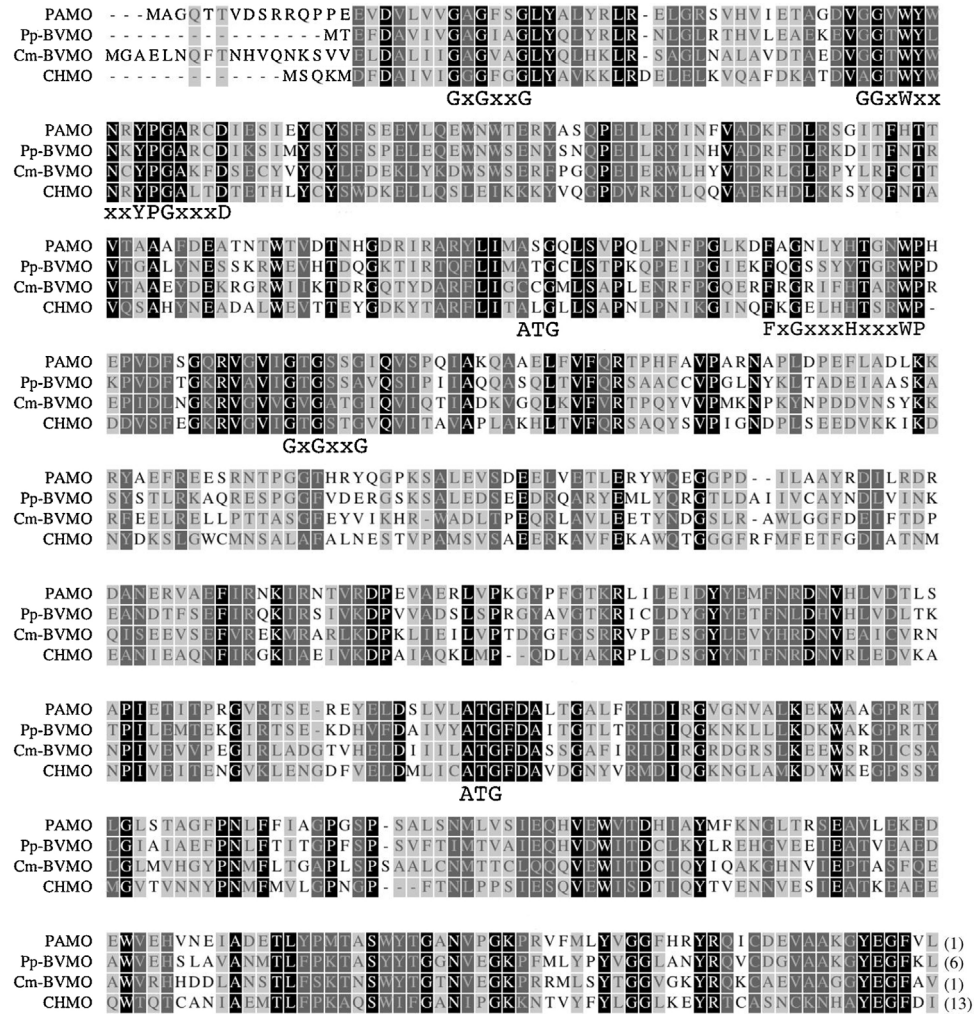


Fig. 1. CLUSTALW Multiple sequence alignment of new BVMOs, PAMO and CHMO. CLUSTALW Multiple sequence alignment of phenylacetone monooxygenase from *Thermobifida fusca* (PAMO), cyclohexanone monooxygenase from *Acinetobacter* NCIMB 9871 (CHMO) and novel BVMOs from *Physcomitrella patens* (Pp-BVMO) and *Cyanidioschyzon merolae* (Cm-BVMO). The two Rossmann fold domains (GxGxx(G/A), ATG) and the two BVMO fingerprint motifs (FxGxxxHxxxW [20] and GGxWxxxxYPGxxxD [24]) are highlighted. Number of omitted terminal residues is in brackets.

of bacterial culture. They were tested in reactions with *rac*-bicyclo[3.2.0]hept-2-en-6-one as substrate, a benchmark molecule for Baeyer–Villiger monooxygenase activity. Conversion of the substrate, as observed by consumption of NADPH, confirmed the nature of the newly discovered enzymes and prompted their further characterization.

3.2. pH and temperature profiles

All recombinant type I BVMOs showed a bell-shaped pH profile in reactions with *rac*-bicyclo[3.2.0]hept-2-en-6-one. Maximum activity was measured at pH 8.5 for Cm-BVMO; at pH 8.0 for Pp-BVMO and Pp-Y160H-BVMO. The latter optimum pH value corresponds to that found for PAMO, whereas pH 7.5 and 9 are the values respectively reported for HAPMO (4-hydroxyacetophenone monooxygenase) and CHMO (cyclohexanone 1,2-monooxygenase; [27]).

Temperature optima were measured between 17 °C and 70 °C. Enzymatic activity of Cm-BVMO increased with temperature up to 70 °C: at this temperature initial enzymatic activity was five-fold higher compared to that measured at 30 °C. The enzyme from *C. merolae* also demonstrated considerable thermostability, since its activity was fully retained in incubation for 1.5 h in the temperature range between 20 °C and 42 °C. Higher incubation temperatures led to inactivation of the enzyme. Considering that the red alga typically grows at 45 °C, this result was not unexpected. Unfolding experiments using the *Thermo*FAD method revealed that the melting temperature (*Tm*) of Cm-BVMO was 56 (±0.5) °C, which is considerably higher than that of most known BVMOs. To our knowledge, the only other thermostable type I BVMO found so far is PAMO, i.e. the enzyme discovered by Fraaije et al. [22] in *Thermobifida fusca*, that exhibits an activity half-life of 24 h at 52 °C and a *Tm* of 61 °C.

Optimal temperatures for Pp-BVMO and Pp-Y160H-BVMO were probed between 17 °C and 40 °C: they resulted around 25 °C and 30 °C respectively for Pp-BVMO and Pp-Y160H-BVMO. Concerning stability, after 1.5 h incubation between 17 °C and 25 °C both enzymes retained their activity. *Tm* of Pp-BVMO and mutant Pp-Y160H-BVMO were 44.0 (±0.5) °C and 43.5 (±0.5) °C respectively. Apparently, the restored histidine residue in the fingerprint motif of the mutant did not significantly influence the melting temperature of the enzyme.

3.3. Substrate scope

To explore the biocatalytic potential of the two eukaryotic BVMOs, a broad range of potential substrates was used in a so-called phosphate assay [24]. In this indirect assay the Baeyer–Villiger

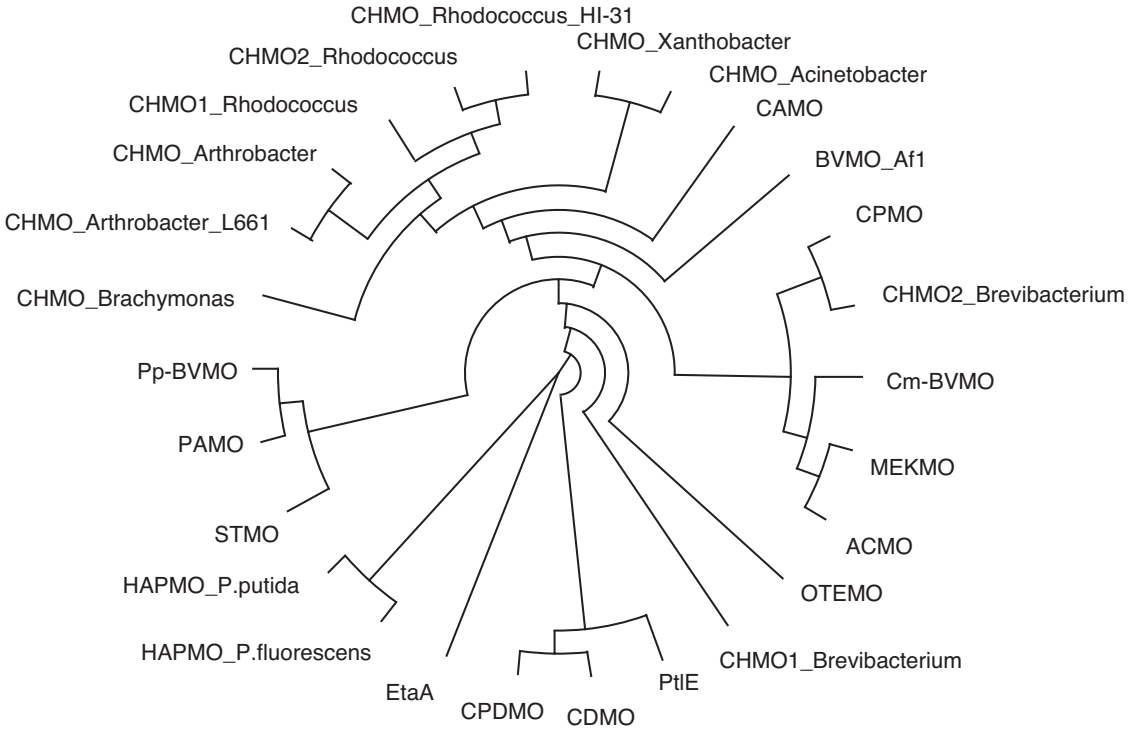


Fig. 2. Phylogenetic analysis of Cm-BVMO, Pp-BVMO and selected BVMOs. MUSCLE multialignment of 26 type I BVMOs (selected as described in Section 3.1) was used as input into MEGA program version 5.0 [40] in order to generate a phylogenetic tree using the neighbor-joining method [41] with an associated bootstrap analysis, based on 1000 replications. The tree was visualized using Dendroscope 3 [42]. Abbreviations and GenBank accession numbers of protein sequences: CHMO_Acinetobacter, CHMO *Acinetobacter* sp. NCIMB 9871 (BAA86293); CHMO1_Brevibacterium, CHMO 1 *Brevibacterium* sp. HCU (AAG01289); CHMO2_Brevibacterium, CHMO 2 *Brevibacterium* sp. HCU (AAG01290); CHMO1_Rhodococcus, CHMO *Rhodococcus* sp. Phi1 (AAN37494); CHMO2_Rhodococcus, CHMO *Rhodococcus* sp. Phi2 (AAN37491); CHMO_Arthrobacter, CHMO *Arthrobacter* sp. BP2 (AAN37479); CHMO_Brachymonas, CHMO *Brachymonas petroleovorans* (AAR99068); CHMO_Xanthobacter, CHMO *Xanthobacter* sp. ZL5 (CAD10801); CPMO, cyclopentanone monooxygenase *Comamonas* sp. NCIMB 9872 (BAC22652); PAMO, phenylacetone monooxygenase *Thermobifida fusca* (PDB: 1W4X_A); HAPMO_P. fluorescens, 4-hydroxyacetophenone monooxygenase *Pseudomonas fluorescens* (AAK54073); STMO, steroid monooxygenase *Rhodococcus rhodochrous* (BAA24454); CDMO, cyclododecanone monooxygenase *Rhodococcus rubber* (AAL14233); EtaA, EtaA *Mycobacterium tuberculosis* H37Rv (CAB06212); CPDMO, cyclopentadecanone monooxygenase *Pseudomonas* sp. HI-70 (BAE93346); ACMO, acetone monooxygenase *Gordania* sp. strain TY-5 (BAF43791); MEKMO, methylethylketone monooxygenases *Pseudomonas veronii* MEK700 (ABI15711); OTEMO, Δ(3)-4,5,5-trimethylcyclopentenylacetyl-coenzyme A monooxygenase *Pseudomonas putida* ATCC 17453 (H3JQW0); CAMO, cycloalkanone monooxygenase *Cylindrocarpon radicicola* ATCC 11011 (AET80001); BVMO_AF1, BVMO *Aspergillus fumigatus* Af293 (NCBI: XM_742067); PtlE, BVMO *Streptomyces avermitilis* MA-4680 (NP_824170); CHMO_Arthrobacter_L661, CHMO *Arthrobacter* sp. L661 (ABQ10653); CHMO_Rhodococcus_HI-31, CHMO *Rhodococcus* sp. HI-31(BAH56677); HAPMO_P. putida, HAPMO *Pseudomonas putida* (ACJ37423).

oxidation is coupled with a regeneration reaction catalyzed by phosphite dehydrogenase (PTDH). PTDH is able to generate one molecule of phosphate from a molecule of phosphite in water, with simultaneous regeneration of one molecule of NADPH. The formed phosphate can be quantified by using a chromogenic reaction that allows spectrophotometric detection of the degree of conversion.

By using this assay, a collection of 46 potential substrates for BVMOs was tested. They were chosen among different classes of molecule, e.g. linear and cyclic aliphatic ketones, aryl and aromatic ketones, steroids, aromatic amines, and aromatic sulphides. The substrate scope obtained for each enzyme is summarized in Table 1.

In general, all the three BVMOs seem to have a broad substrate scope. As expected, they exhibited significant activity against

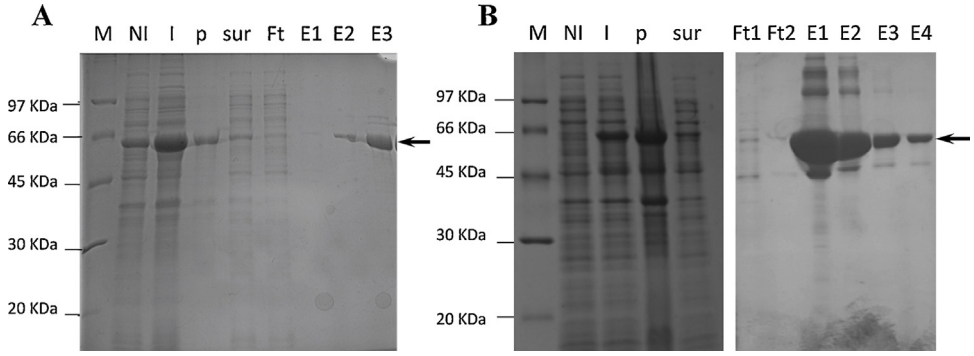


Fig. 3. Expression and purification of His-tagged Pp- and Cm-BVMOs. 12% SDS-PAGE analysis of cell extracts from *E. coli* cells expressing Pp-BVMO (A) and Cm- BVMO (B). Fractions from progressive purification fractions were loaded: protein standard marker (M), total cell extracts from non induced cells (NI) or over night induced cells (ON), pellet fraction (p) and soluble protein fraction (sur), flow trough (Ft) and elution fractions (E).

Table 1
Substrate scope.

Substrate	Cm	Pp	PpY160H
Acetone	+		
Methylketone	+	+	
Methyl vinyl ketone	+	+	+
2-Octanone	++	++	+++
3-Octanone	+	++	+
4-Octanone	+		
2-Dodecanone	+++	++	+++
3-Methyl-2,4-pentanedione	+		+
Cyclobutanone	+	+	+
Cyclopentanone	++	+	++
Cyclohexanone	+		+
Cyclopentadecanone			+
2-Oxocyclohexanecarbonitrile		++	+++
4-Methylcyclohexanone	+	+	+
2-Propylcyclohexanone		+	+
Dihydrocarvone			+
Cyclopropylmethylketone	+		++
Norcamphor		+	+++
Bicycloheptenone	++	++	+
Progesterone			+
Androstenedione			+++
4-Dimethylaminobenzaldehyde		+	+
Nicotin			++
Thioanisole	+		
Benzylethyl sulfide	++	+++	
Benzylphenyl sulfide			+++
Ethionamide		+	
Diphenylmethylthioacetamide			+
Thiacetazone			+
Indole	+		+++
3-Acetylindole			++
5-Mathylfurfural	+	++	+
Benzaldehyde		+	+
Acetophenone		+	++
4-Hydroxyacetophenone		+	+
2,6-Dihydroacetophenone			+++
3-Phenylpentane-2,4-dione	++	++	+++
Phenylacetone	+++	++	+++
4-(4-Hydroxyphenyl)-2-butanone	+++	+++	+
2-Phenylcyclohexanone			+
Benzoin			
Phenindione			
2-Indanone		+	+
1-Indanone		+	+
6-Hydroxy-1-indanone	+		

Observed activities for Cm-BVMO, Pp-BVMO and Pp-Y160H-BVMO, measured by phosphate formation. Activities are indicated as +, ++ or +++ and reflect a 1.2-, 2- or 5-fold increase in phosphate formation, respectively, when compared with uncoupling reactions that lack the tested compound.

rac-bicyclo[3.2.0]hept-2-en-6-one, the above-mentioned typical substrate for type I BVMOs. Good activities were also observed for linear ketones, in particular long-chain alkanones. Phenylacetone and some derivatives also seemed to be easily accepted as substrates, as well as some cyclic ketones like cyclopentanone. It is worth noting that, by directly comparing results collected for Pp-BVMO and for Pp-Y160H-BVMO, the mutant displays higher activity and larger substrate profile. This result is in agreement with the fundamental role reported for the central histidine of the BVMO consensus motif in catalysis and FAD binding. Indeed, in site-directed mutants of 4-hydroxyacetophenone monooxygenase from *Pseudomonas fluorescens* (His296Ala; [19]) and of cyclohexanone monooxygenase from *Acinetobacter* NCIB 9871 (His163Gln; [28]), it was shown that substitution of this residue drastically reduces or nearly abolishes activity.

Other potential substrates tested with the new BVMOs, such as acetone, indanone, phenindione, cyclopentadecanone, benzoin and steroids, were found to be poor or not accepted substrates.

3.4. Steady-state kinetics

Steady-state kinetic parameters of the three recombinant enzymes were determined in 50 mM Tris/HCl using the optimal conditions defined for each enzyme, that are pH 8.0, at 25 °C for Pp- and Pp-H160Y BVMOs and pH 8.5, at 35 °C for Cm-BVMO. All the three enzymes showed a typical Michaelis–Menten behavior. The kinetic parameters k_{cat} and K_M were calculated for a set of identified substrates besides *rac*-bicyclo[3.2.0]hept-2-en-6-one, used in the preliminary assay. Selected substrates were linear aliphatic ketones, cyclic aliphatic ketones and aromatic ketones. In particular, for the linear ketone containing eight carbon atoms, the position of the keto group in the molecule was investigated. Tables 2 and 3 summarize the results obtained for Cm-BVMO, and Pp- and Pp-Y160H BVMOs, respectively.

Cm-BVMO exhibited the best catalytic efficiency against 2-dodecanone (k_{cat}/K_M 90.8 s^{-1} mM^{-1}). Octanones also appeared to be good substrates. In particular, by comparing K_M and k_{cat}/K_M values reported in Table 2, the position of the keto group at C3 seemed to be preferred. By limiting the observation to the positions of the carbonyl function in octanone, it can be noticed that the Michaelis constant is the main parameter influencing the catalytic efficiency, since the k_{cat} values were similar for all investigated positions of the keto groups.

Also for Pp- and Pp-Y160H BVMOs, linear ketones seemed to be very well accepted substrates (Table 3). 2-dodecanone was the one for which the enzymes display the highest catalytic efficiency. Differently from the enzyme from *C. merolae*, the one from *P. patens* preferred the keto group at position 2 of octanone. 4-octanone was not a good substrate for either wt or mutant enzymes, as already observed in the previous substrate-screening assay (Table 1). k_{cat} values, for all the four linear C8-ketones tested, were approximately in the same range of those measured for the algal enzyme. As discussed above in relation to the activity and substrate profile, restoration of the consensus histidine makes the mutant variant of Pp-BVMO more competent for catalysis and faster than the wt form, showing higher turnover number.

All the recombinant enzymes were able to oxidize some substituted aryl ketones, like phenylacetone and 4-(4-hydroxyphenyl)-2-butanone. The latter compound was a good substrate, particularly for Pp- and Pp-Y160H BVMOs. Due to the limited solubility, other substrates such as 4-dimethylaminobenzaldehyde and 3-acetylindole could not be investigated by kinetic analysis. Finally, the affinity for coenzyme NADPH was found to be very high, as a K_M values <5 μM were determined.

3.5. Conversions

Biotransformations using purified enzymes and different types of substrates were investigated using GC and GC-MS analysis in order to detect both substrates and products. PTDH and phosphite were added for NADPH regeneration.

As already mentioned, *rac*-bicyclo[3.2.0]hept-2-en-6-one is a standard probe for testing the biocatalytic potential of type I BVMOs. This conversion is a good example of regiodivergent parallel kinetic resolution (PKR) because the racemic substrate leads to two regioisomeric compounds (−)-(1*S*,5*R*) **2** and (−)-(1*R*,5*S*) **3** (Fig. 4A). One compound originates from the favored Baeyer–Villiger-type oxygen insertion between the more substituted carbon atom and the carbonyl group in a fast reaction; the second lactone is more slowly produced in the chemically disfavored regiochemistry. The two chiral lactones are relevant building blocks for the chemical synthesis of prostaglandins; their production has been therefore studied in conversion experiments by using different strains, i.e. different BVMOs. Some examples of such studies are those regarding CHMO from *Acinetobacter* [29],

Table 2
Steady-state kinetic analysis of Cm-BVMO.

Substrate	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1} mM]
2-Dodecanone	0.004 ± 0.001	0.383 ± 0.018	90.8
Octanal	0.010 ± 0.008	0.252 ± 0.043	25.3
2-Octanone	0.011 ± 0.006	0.250 ± 0.031	22.7
3-Octanone	0.0030 ± 0.0005	0.210 ± 0.006	70
4-Octanone	0.0040 ± 0.0009	0.202 ± 0.006	50.5
4-(4-Hydroxyphenyl)-2-butanone	0.0070 ± 0.0007	0.079 ± 0.002	11.2
Bicyclo[3.2.0]hept-2-en-6-one	0.0040 ± 0.0007	0.085 ± 0.002	21.3
3-Phenylpentane-2,4-dione	0.099 ± 0.026	0.015 ± 0.001	0.2
Phenylacetone	0.097 ± 0.071	0.032 ± 0.006	0.3

cyclopentanone monooxygenase (CPMO) from *Pseudomonas* sp. NCIMB 9872 [30] and 4-hydroxyacetophenone monooxygenase (HAPMO) from *Pseudomonas fluorescens* ACB [5].

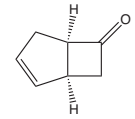
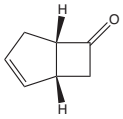
In our experiments, biooxidation of the *rac*-bicyclo[3.2.0]hept-2-en-6-one racemic substrate led to the production of all four possible lactone products by the three BVMOs, and the observed *ee* values suggested poor enantioselectivity toward this racemic substrate. "Normal" lactones for both fast and slow reactions were mainly produced.

The behavior of Cm-BVMO revealed (as shown for HAPMO [5]), a significant preference for the (+)-(1R,5S) **1** enantiomer. Regioselectivity was scarce for both the preferred and non-preferred

Table 3
Steady-state kinetic analysis of Pp- and Pp-Y160H BVMOs.

Substrate	Pp			PpY160H		
	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1} mM]	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1} mM]
2-Dodecanone	0.00040 ± 0.00008	0.096 ± 0.002	240	0.0020 ± 0.0004	0.252 ± 0.011	126
Octanal	0.019 ± 0.009	0.061 ± 0.004	3.2	0.0030 ± 0.0008	0.125 ± 0.006	41.7
2-Octanone	0.0030 ± 0.0008	0.060 ± 0.002	20	0.0030 ± 0.0006	0.204 ± 0.007	68
3-Octanone	0.010 ± 0.005	0.075 ± 0.008	7.5	0.016 ± 0.007	0.123 ± 0.011	7.6
4-Octanone	–	–	–	–	–	–
4-(4-Hydroxyphenyl)-2-butanone	0.002 ± 0.001	0.068 ± 0.004	34	0.0030 ± 0.0007	0.152 ± 0.006	50.7
Bicyclo[3.2.0]hept-2-en-6-one	0.0060 ± 0.0001	0.058 ± 0.0001	9.7	0.016 ± 0.002	0.120 ± 0.005	7.5
Phenylacetone	0.038 ± 0.016	0.077 ± 0.009	2	0.032 ± 0.009	0.146 ± 0.011	4.6

Table 4
Regiodivergent biooxidation by Cm-, Pp- and Pp-Y160H BVMOs.

Substrate	Cm		Pp		PpY160H	
	% Conv ratio	% *ee*	% Conv ratio	% *ee*	% Conv ratio	% *ee*
 Fast reaction	100 73:27	12 (+)-(1*R*,5*S*)**2** 37 (+)-(1*S*,5*R*)**3**	100 76:24	7 (+)-(1*R*,5*S*)**2** 69 (+)-(1*S*,5*R*)**3**	100 72:28	14 (+)-(1*R*,5*S*)**2** 68 (+)-(1*S*,5*R*)**3**
 Slow reaction	100 88:12		66 62:4		100 95:5	

% Conv, percentage yield of conversion; ratio, ratio between produced normal and abnormal lactones; % *ee*, percentage enantiomeric excess values.

substrate enantiomers, that respectively produced 73% (−)-(1*S*,5*R*) **2** versus 27% (+)-(1*S*,5*R*) **3**, and 88% (+)-(1*R*,5*S*) **2** and 12% (+)-(1*R*,5*S*) **3**, enantiomers. All four possible lactone products, in different amounts, were therefore finally obtained (Fig. 4B). The *ee* values of the product mixture resulting from complete conversion were 12% for the "normal" (+)-(1*R*,5*S*) **2** and 37% for the "abnormal" (+)-(1*S*,5*R*) **3**. (Table 4). After 5 h, the racemic substrate was fully converted.

Pp- and Pp-Y160H BVMOs approximately showed the same behavior (Fig. 4C and D) (Table 4). The favored substrate was the (+)-(1*R*,5*S*) **1** enantiomer for both enzymes; they respectively produced 76% and 72% of the expected (−)-(1*S*,5*R*) **2** versus 24% and 28% of the (+)-(1*S*,5*R*) **3**. The mutant enzyme, compared to the wild-type one, exhibited much higher regioselectivity in the slow conversion of the (−)-(1*S*,5*R*) **1** substrate, yielding 95% of the expected lactone and 5% of the unexpected one. In such slow reaction, the WT enzyme yielded only 62% of the expected and 4% unexpected lactone and the conversion was not complete (66%) after 16 h. As for the case of the conversion by Cm-BVMO, *ee* values for the "normal" and "abnormal" enantiomeric products were too low to be considered for a direct biocatalytic application of the new enzymes.

Conversions by Cm-, Pp- and Pp-Y160H BVMOs were also evaluated with other compounds, chosen among those previously identified as substrates. As shown in Table 5, conversion of these ketones resulted in the formation of the expected ester product, obeying the stereo-chemical rule of Baeyer–Villiger reactions where the migrating group is the most substituted one. In particular, 2-octanone, 3-octanone and 4-octanone were fully converted by Cm-BVMO. The mutated BVMO from *Physcomitrella* performed full conversion of 2-octanone and 3-octanone and poor transformation of 4-octanone, whereas the unmodified enzyme was able to oxidize in good amount only the first two linear ketones. Finally phenylacetone was fully converted by all the three enzymes. The recombinant enzymes gave poor or almost no conversion of the cyclic ketones cyclohexanone and cyclopentanone; only Cm-BVMO was able to transform the former molecule.

4. Discussion

BVMOs are currently in the spotlight of industrial biocatalysis for their capability to perform challenging steps in organic chemical processes, such as oxidations of ketones to esters (Baeyer–Villiger oxidations), but also sulfoxidations and other oxidative reactions. Up to 2012, all reported type I BVMOs originated from microbial organisms, particularly actinobacteria and filamentous fungi. Only two BVMOs of eukaryotic origin have been identified and expressed in recombinant form very recently: the BVMOs from the ascomycetes *Cylindrocarpon radicicola* ATCC 11011 [20] and *Aspergillus fumigatus* Af293 [21]. We have identified and selected two new putative BVMOs from the photosynthetic eukaryotes *Cyanidioschizon merolae* and *Physcomitrella patens*.

The two organisms can be certainly considered very uncommon sources for BVMOs, being eukaryotic and photosynthetic. The primitive red alga *C. merolae* 10D is a unicellular organism living in acidic environments (like hot springs), even at pH < 2 and temperature of 45 °C. It is one of the photosynthetic eukaryotes with the most simple cell architecture: its cell does not present a rigid cell wall and contains a single nucleus, a single mitochondrion and a single chloroplast. This clearly offers advantages for studies on these organelles [29,30]. The moss *P. patens* is classified as a no vascular plant, since it differs from higher plants in not having internal vessels [31], and stands in an important phylogenetic position for studying the evolutionary transition from the aquatic environment of algae to the terrestrial one of higher plants. Noticeably, both organisms undergo homologous recombination with a frequency that allows easy targeting of genes for replacement and elimination [32,33]. This allows handy studying of gene function and helps in predicting the physiological role of genes in higher organisms. *P. patens* and *C. merolae* are therefore considered model systems for studying origin and fundamental mechanisms of eukaryotic cells and plant evolution.

We succeeded in producing the identified putative BVMOs in recombinant form, together with a mutant variant of the protein from *Physcomitrella* completely restored in the consensus sequence firstly described. The second BVMO-typifying motif ([A/G]GxWxxxx[F/Y]P[G/M]xxxD), recognized and annotated after the beginning of our work [24], is fully conserved in both proteins. The biocatalytic characterization of the recombinant products could demonstrate their BVMO activity on a broad range of substrates as linear and cyclic aliphatic ketones, aryl and aromatic ketones (Table 1). Among linear ketones (C8 and C12), proven to be the best substrates, 2-dodecanone was the preferred one.

In an overview of the data from the kinetic analysis, the three enzymes displayed different rates of catalysis, depending on the substrate examined (Tables 2 and 3), but in all cases measured k_{cat} values were not higher than 0.4 s^{-1}. Since most natural monooxygenase enzymes display k_{cat} values in the range of 1–100 s^{-1} when tested on ketones among those selected for our analysis [34], we conclude that the new BVMOs (at least as such)

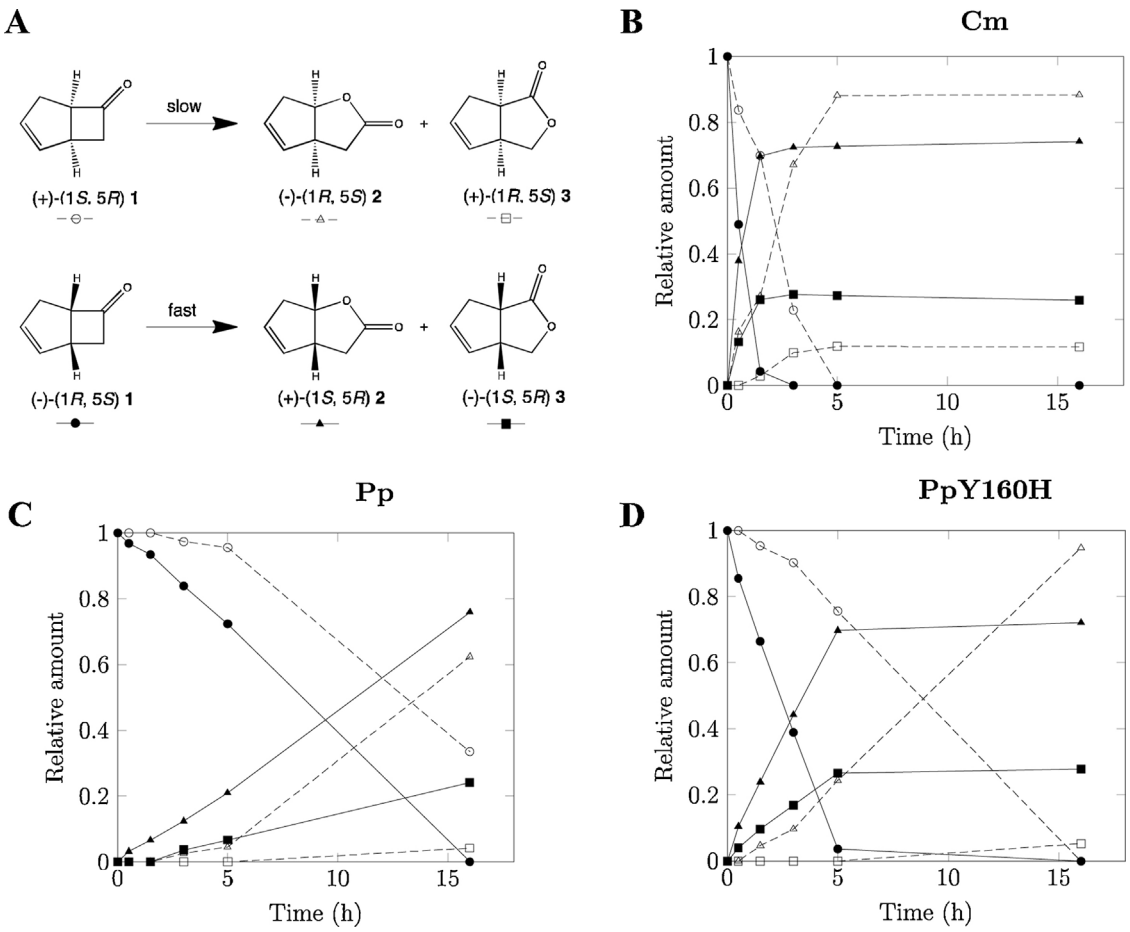
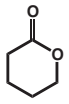
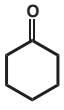
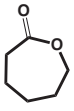
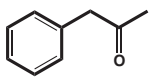
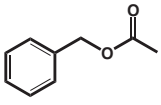
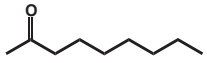
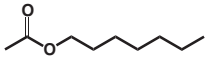
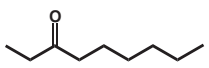
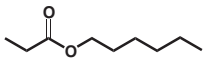
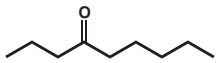
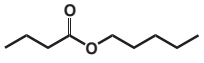


Fig. 4. Biooxidation of racemic bicyclo[3.2.0]hept-2-en-6-one. Possible structures of bicyclo[3.2.0]hept-2-en-6-one isomers and lactones formed by type I BVMO activity (A). Time course of Cm- (B), Pp- (C) and Pp-Y160H- (D) BVMOs catalyzed oxidation of racemic bicyclo[3.2.0]hept-2-en-6-one to 2-oxabicyclo[3.3.0]oct-6-en-3-one (+)-(1S,5R) 2/(−)-(1R,5S) 2 and 3-oxabicyclo[3.3.0]oct-6-en-2-one (−)-(1S,5R) 3/(+)-(1R,5S) 3.

Table 5
Conversion of some identified substrates and corresponding products.

Substrate	Product	Conversion (%)		
		Cm	Pp	PpY160H
		–	17	27
		80	1	3
		100	100	100
		100	90	100
		100	67	100
		100	–	17

seem rather inefficient bio-catalysts. This is also true in terms of enantioselectivity, which was shown to be very poor when probed on the typical substrate racemic bicyclo[3.2.0]hept-2-en-6-one (Table 4), transformed in the four possible lactone.

Nevertheless, some results deserve to be highlighted. As known from the literature, representative BVMOs can be clustered in well-defined groups with different substrate profile. The phylogenetic analysis that we performed (Fig. 2) places the new BVMOs in two distinct positions. The protein from *P. patens* is localized in the clade defined by PAMO and STMO, which transform aromatic (but also linear) ketones; the one from *C. merolae* is clustered together with MEKMO and ACMO, enzymes known for converting linear ketones [35,36]. This grouping is in agreement with the substrate scope obtained for the recombinant enzymes even though, because of their eukaryotic origin, a different biocatalytic behavior could be expected.

The second noteworthy result concerns the central histidine of the motif FxGxxxHxxxWP: this residue, although dispensable for catalysis and not always conserved (see [21] and [24]), has been proposed to be involved in conformational changes occurring along with catalysis [26]. Its fundamental role has been already shown [19,28]. By comparing activities of wild type and mutant Pp-BVMOs, we further confirm here that this histidine is certainly important for enzymatic performance.

Finally, we could show that the enzyme from *Cyanidioschizon* displays a relatively high thermostability by having an apparent melting temperature of 56 °C. This compares favorably with other well-studied BVMOs: CHMO has a *Tm* of 39 °C (near to the value that we measured for Pp-BVMO, i.e. 44 °C), while PAMO has a *Tm* only five degrees higher than that measured for Cm-BVMO [37]. If protein engineering will be considered to boost the enzyme activities in view of a possible re-consideration of their utility, the primary sequence of Cm-BVMO could be a favorable starting point.

In the same perspective, a better characterization of the repertoire of accepted molecules could also be useful. The displayed very low catalytic efficiency suggests that during evolution the two new BVMOs had been subjected to a weak selective pressure. Hence, according to the current view, a potential role as secondary metabolism enzymes can be envisaged [38,39]. Their substrate preferences point toward linear alkanones, structurally similar to side chains of chlorophylls and pheophytins, and to their possible involvement in modifications of photosynthetic pigments. Work is in progress to produce knockout mutants in both the alga and the moss, by exploiting their endogenous homologous recombination. Comparison of null mutants with wild-type strains would help in uncovering the natural substrates of Cm- and Pp-BVMOs in their original biological context.

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